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13. ABSTRACT (Maximum 200

The goal of this project is to develop Bowman-Birk protease inhibitor (BBI), a soybean polypeptide, as a chemopreventive agent for breast cancer. In order to achieve this goal, we proposed the following two specific aims in our original application: (1) using an *in vitro* mammary gland culture system to demonstrate the anti-transformation activity of both BBI and its palmitic acid conjugate (Pal-BBI), and (2) using a mouse model to demonstrate the advantages of Pal-BBI in oral delivery of BBI. During the funding period of this project, we have obtained 4 important results. First, we developed a new method to conjugate fatty acid to BBI via the intramolecular disulfide bonds in the polypeptide. Second, we demonstrated that BBI can prevent the transformation of mammary glands induced by the treatment of a chemical carcinogen, 7,12-dimethylben[a]anthracene (DMBA). Third, we demonstrated that Pal-BBI is a better chemopreventive agent than BBI in mammary gland transformation assay. Finally, we demonstrated that Pal-BBI is more stable than the native BBI in mouse gastrointestinal tract. However, we failed to demonstrate that Pal-BBI could significantly improve the oral bioavailability in mouse model. Results from our studies will be useful for developing effective diets or chemopreventive agents for the prevention of breast cancer in the future.

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FOREWORD

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PI - Signature   Date   8/12/99

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(5) INTRODUCTION

This is the final report on the project, "Breast Cancer Prevention by a Soybean Protein", which was originally proposed for the period from July 15, 1996 to July 14, 1998. However, due to unforeseen problems in mammary gland culture, the progress of this project was delayed and, subsequently, a no-cost one-year extension of this project to July 15, 1999 was granted. In the original application, we planned to investigate the chemopreventive effect of Bowman-Birk protease inhibitor (BBI), which is an 8 kDa polypeptide isolated from soybean, on breast cancer formation. It has been shown that BBI can suppress cellular transformation and carcinogenesis *in vitro* and reduce incidence of a variety of malignant tumors *in vivo* (1). Even though epidemiological studies suggest that vegetarians, especially those who consume legumes as the major source of food, have a significantly lower incidence of breast cancer (2,3), the chemopreventive effect of BBI on breast cancer has not been demonstrated. We have recently developed a method of conjugating BBI to a fatty acid, palmitic acid, and the product, Pal-BBI, appeared to have increased lipophilicity. Therefore, we proposed to use Pal-BBI as a lipophilic polypeptide to investigate the gastrointestinal (GI) absorption of BBI and to develop an oral delivery formulation for breast cancer prevention.

This final report is presented in two sections, corresponding to the two technical objectives in the Statement of Work in the original application: (a) to demonstrate breast cancer preventive effect of BBI and Pal-BBI in cultured mouse mammary glands, and (b) to demonstrate the GI absorption of Pal-BBI in mice.

(6) BODY

I. Technical Objective 1

A. Preparation of Pal-BBI (Objective 1, Task 1)

In our original proposal, we planned to prepare Pal-BBI by using the procedure described in our previous report, which involved the preparation of a 2-pyridyl 3-propionate disulfide succinimidyl ester (SPDP)-modified BBI and subsequently SPDP-modified BBI was reduced to generate sulfhydryl groups for the conjugation reaction. We recognized that several shortcomings were associated with this procedure. First, it is difficult to control the number of SPDP incorporated into each BBI molecule. The number of SPDP, and the subsequent sulfhydryl groups, per each BBI in this preparation represents only an average of a mixture product. Furthermore, the reduced form of Pal-BBI prepared from this procedure are chemically modified BBI molecules rather than the native BBI. In addition, the yield of the final product was inconsistent and was usually very low because this method involves many steps for the preparation of Pal-BBI.

In order to overcome these problems, which is critical for the success of this project in the future, we have developed a new method for the preparation of Pal-BBI. In this method, the endogenous disulfide bonds in BBI molecule will be selectively reduced (4), and conjugated with N-palmitoyl cysteinyl 2-pyridyl disulfide (CPD) as described previously (5) to produce the final Pal-BBI. When reduced to remove the fatty acid moieties, this conjugate product will regenerate the original BBI molecule rather than derivatives. The new method of Pal-BBI preparation, as well as its uptake by cultured human enterocyte-like Caco-2 cells, has been described in the 1st Annual Report, 1997.

B.   Mammary gland culture (Objective 1, Task 2)

In order to reduce the number of animal used in each experiment, we tried to use more than just the second pair (thoracic) of mammary glands in each mouse as described in the literature (6,7). We found that the first pair (cervical) of mammary glands were also suitable to be used for the assay (see Table 1 in 2nd Annual Report, 1998). Cervical mammary glands of the mouse are easy to be dissected because they are not covered by other tissues and, unlike the mammary glands of the third pair and below, do not attach with a large fat tissue. By using the first and the second pairs, we were able to obtain four glands per each mouse. However, as suggested by the reviewer of our 1st Annual Report in 1997, in all of the transformation experiments, the mammary glands from each mouse were split equally into the treated and the control groups so that each group consists of same number of the cervical and thoracic, as well as right and left, mammary glands.

C.   Transformation assay (Objective 1, Task 3)

Because the range of DMBA concentrations used in carcinogenesis assays reported by others was very large, experiments were carried out to determine the optimal concentration to be used in our culture system. Concentrations of DMBA were tested from 0.001 to 3.0 µg/ml. It was found that concentrations below 0.1 µg/ml of DMBA did not show any mutagenic effect. On the other hand, 3 µg/ml of DMBA was toxic to cultured mammary glands (see Table 2, 2nd Annual Report). Therefore, a concentration of 2 µg/ml of DMBA in DMSO was finally selected for the assay system.

Because we used both the first (cervical) and the second (thoracic) pairs of mammary glands in our tests, concerns were raised whether there was a difference between these two sets of glands in the transformation assay. To clarify this issue, we compared results obtained from cervical and thoracic mammary glands from same group of mice in their responses to DMBA-induced transformation. The differences between these two sets of glands in DMBA-induced transformation and BBI-mediated chemoprevention are statistically insignificant (see Table 1 in 2nd Annual Report, 1998).

In a typical transformation assay, cultured mammary glands were divided in two groups, i.e., the control and the transformation groups. Mammary glands in the control group were treated with only DMSO, the solvent of DMBA; and those in the transformation group were treated with 2 µg/ml of DMBA in DMSO. Mammary glands were treated on day 3 and 4 only and then moved to the promoting medium and lactogenic medium for a total of 10 days before changed to regression medium. After 14 days in the regression medium, mammary glands were fixed in ethanol:acetic acid, 3:1, for 60 min, washed in 70% ethanol for 15 min, double-distilled water for 5 min, and then stained in Alum Carmine. After overnight staining, fixed glands were washed consecutively with 70%, 95%, and 100% ethanol each for 15 min, and then dehydrated in toluene for 15 min. The final mammary glands were mounted on slides and examined under a dissecting microscope.

As reported by others (8,9,10), control mammary glands maintained only the regression ductal structures and lobulo-alveolar vestiges. However, in DMBA-treated glands, numerous nodule-like alveolar lesions (NLAL) were found. Under a higher magnification of the microscope, NLAL could be seen as densely stained nodules with an irregular shape (see Fig. 2 in 1st Annual Report, 1997). At 2 µg/ml of DMBA, approximately 90% of viable glands showed the presence of NLAL. The number of glands with NLAL divided by the total number of viable glands in a specific treatment group was considered as the incidence of transformation. The preventive efficiency of BBI or Pal-BBI was expressed as:

Preventive efficiency = [1- (Incidence of transformation/Incidence by DMBA only)]x100%

D.   Comparison the chemopreventive effects of BBI and Pal-BBI in DMBA-induced transformation of cultured mouse mammary glands (Object 1, Task 4, 5, 6, and 7)

When BBI and Pal-BBI were tested for their chemopreventive effects, they were included in both the promoting and lactogenic medium, but not in the regression medium. A dose of 2 µg/ml was chosen for DMBA because it was the highest concentration without significant toxicity. In addition, 2 µg/ml of DMBA also showed a higher transformation incidence than that of either 3 or 4 µg/ml of DMBA in the assay, possibly due to a low toxicity. The presence of BBI in the culture medium from day 3 to day 10 was capable of preventing DMBA-induced transformation in cultured mammary glands. A BBI concentration of 20 µg/ml was chosen because it is the lowest concentration of BBI showing a significant prevention on DMBA-induced transformation. At this concentration, Pal-BBI and BBI have an identical chemopreventive effect. This result indicates that Pal-BBI is at least as effective as BBI in the prevention of the DMBA-induced transformation of mammary glands (see Table 2 in 2nd Annual Report, 1998).

E.   Comparison of BBI and Pal-BBI with different conditions (Objective 1, Task 8)

We have demonstrated before that the absorption of Pal-BBI to cultured cells is higher than that of BBI (5). Therefore, Pal-BBI may be more effective than BBI in the prevention of mammary gland transformation if the length of the treatment is shortened. Several experiments were performed in which the schedule of BBI and Pal-BBI treatment has been changed from the standard day 3-10 schedule to day 0-3, day 3-4, day 4-10, and day 7-17. These different time schedules were based on previous reports by others on the studies of BBI on the transformation of C3H10T1/2 cells induced by chemical carcinogens (11). Because of a large number of groups was used, the number of mammary glands in each group was relatively small. Therefore, this experiment has been repeated 5 times during the last few months. The compiled results from three successful experiments are presented in Table 1. These results clearly demonstrate that Pal-BBI is better than BBI in the prevention of transformation when they are presented in the culture medium either for a shorter time or before the treatment of carcinogen, DMBA. The most striking difference was in the treatment from day 3 to day 4 only, i.e., BBI or Pal-BBI was present in the medium only during the treatment of DMBA. In this group, Pal-BBI clearly showed a statically significant decrease in transformation incidence while BBI did not. This difference is most likely due to the high absorption and retention of Pal-BBI by the mammary glands in the culture and, therefore, it will be present in the mammary glands even after the Pal-BBI-containing medium has been removed.

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TABLE 1. Comparison of Preventive Effects of BBI and Pal-BBI in Mammary Gland Transformation Assay with Different treatment Schedules

Group#	Exp.#	Glands with NLAL / Total viable glands	Sum	% Incidence	% Prevention	p-value (Chi-square test) (versus Group# 2)
1. Control	1	1/9	3/27	11%		
	2	1/10				
	3	1/8				
2. DMBA 2 µg/ml, day 3-4	1	8/9	23/26	88.5%		
	2	9/10				
	3	6/7				
3. DMBA BBI, day 0-3	1	8/10	26/30	86.7%	2.0%	0.83949
	2	8/10				
	3	10/10				
4. DMBA BBI, day 3-4	1	10/14	27/34	79.4%	10.3%	0.35129
	2	7/10				
	3	10/10				
5. DMBA BBI, day 4-10	1	5/10	17/30	56.7%	35.9%	0.00019
	2	7/10				
	3	5/10				
6. DMBA BBI, day 7-17	1	8/10	23/30	76.7%	13.3%	0.25041
	2	7/10				
	3	8/10				
7. DMBA Pal-BBI, day 0-3	1	10/16	21/31	67.7%	23.5%	0.06333
	2	3/5				
	3	8/10				
8. DMBA Pal-BBI, day 3-4	1	13/17	21/35	60.0%	32.2%	0.00047
	2	5/10				
	3	3/8				
9. DMBA Pal-BBI, day 4-10	1	4/10	14/34	41.2%	53.4%	0.00019
	2	4/10				
	3	6/14				
10. DMBA Pal-BBI, day 7-17	1	7/10	22/30	73.3%	17.2%	0.15529
	2	8/10				
	3	7/10				

II.   Technical Objective 2

A.   Preliminary Studies of Oral delivery of BBI and Pal-BBI (Objective 2, Task 1, 2, 3)

BBI and Pal-BBI were iodinated with ^{125}I using a modified chloramine T method. Female CF-1 mice, 2 to 3 weeks old, weighing 20-25 g each, were used in these studies. The mice were fasted for 16 hr prior to all of the experiments described here. A dose of 3 mg/kg of labeled BBI or Pal-BBI in 0.2 ml, which was about 1×10^6 cpm of radioactivity, was given to each mouse orally via a gavage needle. Formulations used included PBS, olive oil, and Intralipid (Pharmacia). At various time intervals after gavaging feeding, 3 animals from each group were sacrificed and their blood (200 ml), kidneys, lungs, liver, stomach, intestine and colon were collected. After rinsed extensively in ice-cold phosphate buffered saline (PBS), the radioactivity in each organ was counted in a gamma counter. The weights of the organs were recorded and used to adjust the concentration of BBI or Pal-BBI in each organ. The nature of the accumulated radioactivity in the blood of the animals was determined by Sephadex G-50 gel filtration column. Briefly, 0.2 ml of blood was diluted with 0.8 ml of water and centrifuged at 3000 rpm for 5 min. The supernatant was then eluted from the column using PBS. Fractions (1 ml) were collected and the radioactivity was determined. Results from the blood analysis demonstrated that intact BBI was detected only in the blood of mice received the olive oil, but not in PBS, formulation. A comparison of the olive oil formulation for the oral delivery of BBI and Pal-BBI indicated that the stomach retention was higher for Pal-BBI than BBI in the first 6 hr post-feeding. Similarly, the biodistribution of Pal-BBI was also higher than that of BBI in all tissues except the kidneys within first 6 hr. However, no significant difference was detected after 8 hr post-feeding (see Fig. 3 and 4 in 1st Annual Report).

B.   Biodistribution and pharmacokinetics of BBI and Pal-BBI in Balb/c mice (Objective 2, Task 6)

The biodistribution and pharmacokinetic studies were carried out in Balb/c mice, rather than in CF-1 mice as proposed in our original proposal. This change was suggested by the reviewers of the 1st Annual Report, 1997, in order to be consistent with the same strain of mouse used in the transformation assays. To verify previous results, a study was carried out to compare the pharmacokinetics and biodistribution of intravenously injected BBI and Pal-BBI in Balb/c with data obtained previously from CF-1 mice. It was found that *iv*-injected Pal-BBI in BALB/c mice can achieve (a) a prolonged plasma half-life of Pal-BBI, (b) an increase of liver absorption of BBI, and (c) a decrease of kidney elimination of BBI. These results are identical to those obtained in CF-1 mice as described in our previous report (12).

Our hypothesis was that an enhancement of the GI absorption of BBI and, subsequently, a targeting of this polypeptide to fat tissues such as mammary glands, could be achieved by increasing its lipophilicity by conjugation with fatty acid. Therefore, we also isolated breast tissues from mice and determined the distribution of BBI or Pal-BBI. As shown in Table 2, there was no significant difference in breast-associated radioactivity when mice were treated with ^{125}I-BBI or ^{125}I-Pal-BBI, i.e., 0.7% or 1.0% of total radioactivity, respectively. This finding is disappointing, and requires further investigation in the future.

TABLE 2. **Biodistribution of Intravenously Injected BBI or Pal-BBI in Balb/c Mice**

Treatment	Time (hr)	% Total injected radioactivity per organ ($\pm$ sd, n=3)			
		Blood	Liver	Kidneys	Breast tissue
^{125}I-BBI	0.5	7.9 $\pm$ 1.7	1.9 $\pm$ 0.3	23.4 $\pm$ 3.0	0.7 $\pm$ 0.1
	1.0	6.7 $\pm$ 1.2	2.0 $\pm$ 0.3	9.9 $\pm$ 3.1	0.7 $\pm$ 0.1
	2.5	5.4 $\pm$ 1.5	1.6 $\pm$ 0.2	2.5 $\pm$ 0.6	0.7 $\pm$ 0.2
[125]I-Pal-BBI	0.5	42.7 $\pm$ 2.9	26.6 $\pm$ 0.7	3.6 $\pm$ 0.1	1.1 $\pm$ 0.1
	1.0	29.6 $\pm$ 6.6	22.3 $\pm$ 2.5	2.9 $\pm$ 0.4	1.0 $\pm$ 0.1
	2.5	19.0 $\pm$ 4.4	9.1 $\pm$ 2.3	2.0 $\pm$ 0.3	1.2 $\pm$ 0.1

C.   Oral delivery of BBI and Pal-BBI in Balb/c mice (Task 4, 5 and 6)

Female Balb/c mice, 7-8 weeks old, were fasted for 16-hr and, subsequently, were fed with either ^{125}I-BBI or ^{125}I-Pal-BBI using a gavage needle. The dose and radioactivity of BBI and Pal-BBI were identical, i.e., 3 mg/kg in 1% Intralipid-PBS and 5×10^6 cpm per mouse. Animals were sacrificed at 0.5, 1.5 and 3 hr after the feeding, and the following organs were removed and counted in a gamma counter: blood, liver, kidneys, small intestine, large intestine, and stomach. There was no statistically significant difference between BBI and Pal-BBI in blood, kidneys and liver, because the amounts localized in these organs were too low to be determined. However, there were significant differences in the localization in the GI tract. The retention of Pal-BBI in stomach was strikingly longer than that of BBI. The prolonged stomach retention resulted in an increase of the GI transit time of Pal-BBI as indicated in the time-dependence of the small and large intestine-associated radioactivity (see Table 3 in 2nd Annual Report, 1998).

D.   Analysis of the stomach-associated BBI and Pal-BBI (Task 6).

Since the most noticeable difference between the orally administered BBI and Pal-BBI was the stomach retention, we further investigated the composition of the radioactivity in the stomach. In this study, stomachs of mice that were fed orally with either ^{125}I-BBI or ^{125}I-Pal-BBI, were cut to open and stomach-associated mucosal fluid was collected. The mucosal fluid was diluted with PBS and subsequently precipitated with 10% trichloroacetic acid (TCA). The TCA-precipitate fractions were considered as intact polypeptides and the TCA-soluble fractions as degradation products. The difference between the intact polypeptide in the stomach of Pal-BBI fed mice and of BBI fed mice was even more striking than the total radioactivity. When expressed as the amount of intact polypeptide in the stomach, Pal-BBI was 34-, 17-, and 3-fold higher than BBI in 0.5, 1.5, and 3 hr, respectively (see Table 3 in 2nd Annual Report, 1998).

E.   Identification of Pal-BBI or BBI in isolated tissues from Pal-BBI administered mice
(Objective 2, Task 7)

Because the GI absorption of orally administered ^{125}I-Pal-BBI in mice was very
low, the identification of radioactive species in isolated tissues as intact Pal-BBI or
regenerated BBI was unsuccessful. We were disappointed that an increase of gastric
stability and a prolongation of intestinal transit time by lipidization did not increase
significantly the oral bioavailability of BBI. The causes of the low GI absorption of Pal-
BBI in mice will be discussed in Conclusion section.

(7)   KEY RESEARCH ACCOMPLISHMENTS

- New method for peptide-fatty acid conjugation.
- Demonstration of chemopreventive activity of BBI in mammary glands.
- Demonstration of stability of Pal-BBI in the GI tract.
- Demonstration of the superiority of Pal-BBI in chemoprevention.

(8)   REPORTABLE OUTCOMES

- Manuscripts, abstracts, presentations:

Results obtained from the study of pharmacokinetics and biodistribution of pal-BBI in mice
has been presented in several international meetings, including: 24th International Symposium on
Controlled Release of Bioactive Materials, 1997 (with abstract), 43rd Alfred Benzon Symposium
on Peptide and Protein Drug Delivery, 1997, and 2nd Annual IIR Conference on Drug Delivery
Systems, 1998.

Results obtained from the study of chemopreventive effects of BBI and Pal-BBI in cultured
mammary glands will be presented in Era of Hope Meeting, June 2000. We are currently
preparing a manuscript on the study of BBI and Pal-BBI as chemopreventive agents in mammary
gland transformation. A complete manuscript will be sent to MCMR when it is finished.

- Funding applied for based on work supported by this award

No grant application has been submitted yet due to the delay of generating results from this
project. However, since promising results have been obtained from this study, we intend to
continue to investigate the potential role of BBI in breast cancer prevention.

- Research opportunities received on experience supported by this award

Prof. Xiangtang Du, Visiting Scholar from Henan Medical University, China, learned the
technique of mammary gland culture from our laboratory. He returned to China this month and is
planning to use this technique to pursue breast cancer research.

(9)   CONCLUSION

To our knowledge, our study demonstrated for the first time that BBI can preventive the
transformation of cultured mammary glands induced by carcinogen. This finding suggests that the
preventive effect of soybeans on breast cancer is possibly due to BBI content. Currently, most
studies of the chemopreventive effect of soybean on breast cancer are focused on isoflavones,
antagonists of estrogen (13); however, there is no direct evidence to support the effectiveness of

isoflavones in cancer prevention. Therefore, our finding provides an alternative route to understand the correlation between the incidence of breast cancer and soybean diets. This is especially important for other types of cancer, because many of them such as prostate cancer appear to be preventable by soybean diets but are not estrogen-dependent.

Furthermore, we demonstrated that by conjugating BBI with a fatty acid, palmitic acid, the lipidized BBI (Pal-BBI) possesses a favorable pharmacokinetic parameters than the original polypeptide, i.e., a prolonged plasma half-life, a higher tissue absorption, and an increased GI stability. In addition, we also demonstrated that Pal-BBI is a better chemopreventive agent than native BBI in mammary gland transformation assay. However, we were unable to demonstrated that Pal-BBI, as we hypothesized in the original application, can be absorbed by the gastrointestinal tract. Several reasons may have caused the low GI absorption of Pal-BBI. Factors that should be considered in the future studies of oral delivery of lipidized BBI for breast cancer prevention include the number of fatty acid per each BBI molecule, the chain length of the fatty acid, and use of effective formulations to further enhance the intestinal epithelial transport.

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(11) APPENDEICES – One publication

(12) FINAL REPORTS

A.   Publications:

Shen, W.C., Wang, J., and Shen, D., Reversible lipidization of polypeptides in drug delivery. Proceed. Intl Sym. Control. Rel. Bioact. Mater. 24:202-203 (1997). (Abstract).

Wang, J. and Shen, W.C., Gastric retention and stability of lipidized Bowman-Birk protease inhibitor in mice. (Submitted and revised)

Du, X. and Shen, W.C., Bowman-Birk protease inhibitor and its palmitic acid conjugate prevent 7,12-dimethylbenz[a]anthracene-induced transformation in cultured mouse mammary glands. (Submitted)

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